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13. ABSTRACT (Maximum 200 Words)

Numerous studies indicate that these Rho GTPases are often either overexpressed or hyperactive in breast cancer tissue. Previously, we cloned and characterized a novel human target of Rho, designated Rhophilin(RL). To explore the function and localization of this protein, we raised an antibody to Rhophilin-2 and successfully utilized it to detect endogenous Rhophilin. In an attempt to further understand the biological function of Rhophilin-2, we performed yeast two-hybrid library screen with the C-terminus of Rhophilin-2 and identified α-actinin-4. Using glutathione S-transferase capture assays we confirmed that Rhophilin-2 interacted with α-actinin-4 and also with α-actinin-1. Mapping of the Rhophilin-2-α-actinin interaction revealed it required the region of Rhophilin-2 spanning amino acids 420-454. The region of α-actinin that interacted with Rhophilin-2 did not involve the C-terminal PDZ-binding sequence but mapped to the EF-hands. However, using immunoprecipitation Rhophilin-2 and α-actinin-4 were not found to interact *in vivo*. In light of Rhophilin-2 strong binding to just the EF-hands of α-actinin, we tested a structurally EF-hand containing molecule, MRLC2. We found that Rhophilin-2 interacted with MRLC2 both *in vitro* and *in vivo*. These results suggest that Rhophilin-2 may function in a Rho pathway to regulate the distribution and activity of MRLC2 and other EF-hand containing molecules.

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I. Introduction

Rho, a member of the Rho GTPase family, regulates stress fiber formation, cell motility, cytokinesis, and kinase signaling pathways. Rho signaling pathways are important for cancer progression since they are involved in controlling cell adhesion, anchorage-independent growth, and cell cycle progression. While no mutations in Rho GTPases have been found in breast or other cancers, numerous studies indicate that these GTPases are often either overexpressed or hyperactive in breast cancer tissue (1). A number of downstream effector proteins of Rho have been identified that are involved in Rho-mediated effects (2). We identified a ubiquitously expressed human RhoA-binding protein, designated Rhophilin-2 (3). Rhophilin-2 shows 40% amino acid similarity to human Rhophilin-1 and contains an N-terminal Rho-binding, a central Bro1-like, and a C-terminal PDZ domain. GST-capture experiments revealed that Rhophilin-1 and Rhophilin-2 interacted with both GDP- and GTP-bound RhoA *in vitro*. Despite the ability of Rhophilin-1 and Rhophilin-2 to interact with RhoA in a nucleotide independent fashion, Rho-induced serum response element (SRE) transcriptional activity was not altered by expression of either of these molecules. Although Rhophilin-2-expressing HeLa cells showed a loss of actin stress fibers, Rhophilin-1 expression had no noticeable effect on the actin cytoskeleton. Coexpression of Rhophilin-2 with a constitutively active RhoA mutant reversed the disassembly phenotype, in which coexpressing cells were more spread and less contracted than Rho alone expressing cells. Expression of various Rhophilin-2 deletion and point mutants containing the N-terminal RhoA-binding domain but lacking other regions suggested that the disassembly of F-actin stress fibers was not simply caused by Rho sequestration. Our results with a minor splice variant of Rhophilin-2 suggested that it was involved in protein trafficking and cytokinesis. While studies published from our laboratory and presented here suggest a role of the Rhophilin-2 in actin dynamics, additional evidence suggests a likely role in other pathways.

II. Body

This study originally proposed to uncover the function of RL in cytokinesis and protein trafficking. Since this original grant proposal was accepted in July 2000, it has taken a major turn in focus and also involved a change in the PI. This proposal was based on studies with a new Rho GTPase effector molecule, RL that was discovered in our laboratory (3). Specifically, these hypotheses were based on RL overexpression studies showing that the protein localized to vesicles and caused defects in cytokinesis examining the localization and function in cells, respectively. Since this time, we have now found that RL is a lowly expressed splice variant of a larger protein we now call Rhophilin-2 (Fig. 1). This larger form Rhophilin-2, and not RL appears to be ubiquitously expressed and represents the physiological gene product.
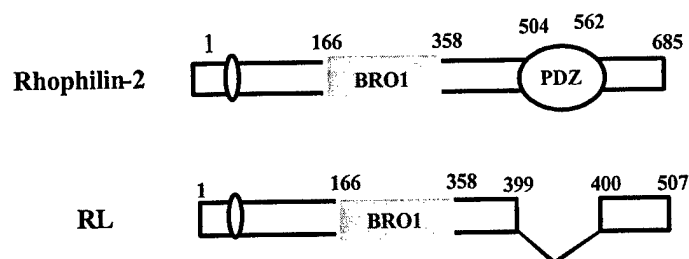
Fig. 1. Structure of Rhophilin-2 and RL.
The Rho-binding domain is shown by the small oval. The striped central region denotes the BRO1 domain. The oval in the C-terminus of Rhophilin-2 shows the PDZ domain. The numbers above indicate the amino acids positions of the domains. Both RL and Rhophilin-2 are derived from the same gene and represent mRNA spliced forms. Only Rhophilin-2 and not RL are expressed in cells and tissues.

RL is identical to Rhophilin-2 within the coding and 3'-untranslated region, except that it is missing sequences that would encode the 178 amino acid regions at the carboxyl-terminus of Rhophilin-2 (Fig. 1). Further analysis of Rhophilin-2 genomic sequence confirmed that these two different mRNA were derived from alternative splicing, since RL was missing a region corresponding to five exons from the Rhophilin-2 coding sequence (data not shown). Reverse transcriptase-PCR of RNA from a variety of cell and tissue sources using oligonucleotide primers that could amplify both Rhophilin-2 and RL mRNAs was used to determine if both isoforms were expressed. We found that the Rhophilin-2 transcript was ubiquitously expressed in all cells/tissues examined including breast, liver and ovarian cancer cells, while a smaller transcript presumed to encode RL was found only in ovarian cancer cells and only in trace amounts (data not shown).

In light of these findings we have focused our attention on determining the function of Rhophlin-2. Using indirect immunofluorescence, we looked for the cellular distribution and biological effect of overexpressed N-terminal FLAG epitope-tagged RL in primary epithelial cells. In primary keratinocytes, the epitope-tagged Rhophilin-2 was uniformly distributed throughout the cytoplasm (Fig. 2A). An examination of endogenous F-actin in cells overexpressing Rhophilin-2, using Texas Red-conjugated phalloidin staining, showed a marked change in F-actin distribution. The normal distribution of F-actin in primary keratinocytes is concentrated mainly in adherens junctions, stress fibers and cortical membrane regions (Fig. 2B). However, in many Rhophilin-2-expressing cells, this normal F-actin staining pattern was significantly eliminated resulting in the loss of F-actin at adherens junctions (Fig. 2B).

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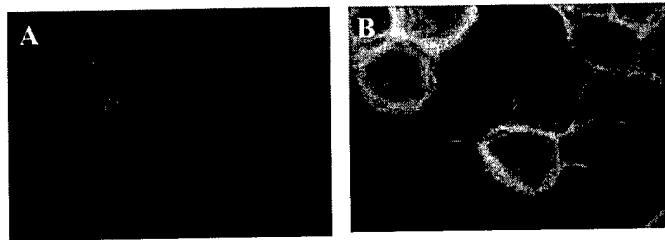
Fig. 2. Overexpression of Rhophilin-2 causes loss of adherens junctions in human keratinocytes.
An N-terminal FLAG epitope-tagged Rhophilin-2 construct was transfected into primary keratinocytes (A, B). The cells were fixed 24 hours post-transfection and processed for indirect immunofluorescence using an anti-FLAG monoclonal antibody, followed by FITC-conjugated goat anti-mouse secondary antibody to detect Rhophilin-2 protein expression (A). Cells were co-stained for F-actin using Texas Red conjugated-phalloidin (B).

Previously we found that RL induced a multinucleated phenotype. As proposed in Aim #2, we tested whether the wild type Rhophilin-2 expression vector and a number of deletion mutants induced the multinucleated phenotype. Specifically, we found that while wild type protein had no effect on cytokinesis, overexpression of mutants within the C-terminus did cause alterations in cytokinesis (Fig. 3). We have validated this phenotype by cell counting and using DAPI staining and found that cells expressing Rhophilin-2-Δ2 and Rhophilin-2-Δ3 showed the presence of lobular nuclei in approximately 40% of the expressing cells (Fig. 3). The exact functional significance of this phenotype is not clear but it may represent a mild defect in cytokinesis. However, using mutants of Rhophilin-2, we were able to dissociate the effect on actin disassembly from the effect on cytokinesis. Specifically, full length Rhophilin-2 caused actin disassembly, while only C-terminal mutants of Rhophilin-2 caused alterations in cytokinesis (Fig. 3).
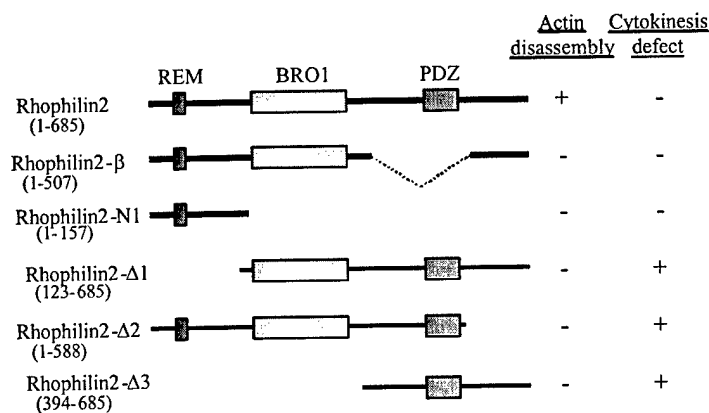
Fig. 3. Role of Rhophilin-2 and its mutants on F-actin disassembly and cytokinesis.
The structure of Rhophilin-2 and mutant constructs are shown. All constructs used in the transient transfection studies contain a FLAG epitope tag at the N-terminus. F-actin disassembly was scored as positive if greater than 80% of the transfected cells showed a lack of stress fibers as detected by phalloidin staining. Cytokinesis was observed by DAPI staining.

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Since our studies suggested an important role for the C-terminus of Rhophilin-2 in altering both the normal F-actin cytoskeleton (3), we predicted that this region might directly or indirectly bind actin or an actin-associated protein within membrane lamellipodia. To specifically address this hypothesis, we performed a yeast two-hybrid screen of a mouse kidney cDNA library for proteins that could interact with the C-terminus of Rhophilin-2. From a screen of approximately 200,000 recombinants, a single positive clone was detected. DNA sequencing revealed that this positive clone was in frame with the GAL-4 activation domain and encoded amino acids 531-912 of the mouse homologue of α-actinin-4. Retesting of this positive clone confirmed the specificity of this interaction, in which the α-actinin-4 clone showed a specific interaction with the C-terminus of Rhophilin-2 and not with other unrelated bait constructs (Fig. 4A). α-actinin-4 consists of an N-terminal actin-binding domain, four central spectrin-like repeats, four C-terminal EF-hands, and a C-terminal type I PDZ consensus sequence (Fig. 4B). These results suggest that α-actinin-4 and other isoforms might interact with Rhophilin-2.
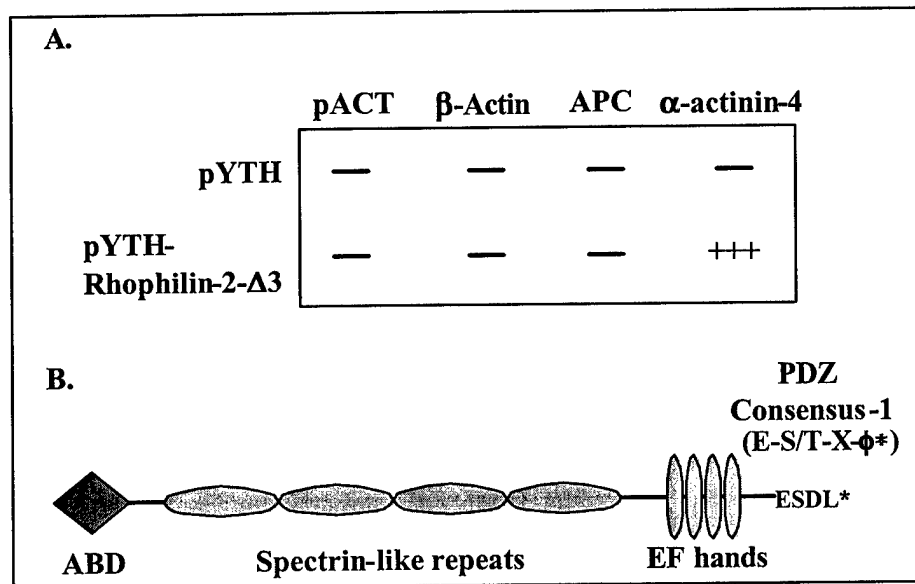

Fig. 4. α-actinin is a binding partner of Rhophilin-2 in yeast two-hybrid tests.
pYTH9 GAL4-DNA binding constructs were generated for the wild-type Rhophilin-2-Δ3 (amino acids 394-885) containing the PDZ domain and then integrated into Y190 yeast cells. The Y190:pYTH9-Rhophilin-2-Δ3 yeast strain was used to screen a mouse kidney library, which identified α-actinin-4 (amino acid residues 531- 912) as a binding partner. Additional yeast two-hybrid studies using β-galactosidase as the reporter were used to confirm the interaction. These tests involved using pACT, β-actin, APC, and α-actinin-4, with empty vector (pYTH9), and pYTH9-Rhophilin-2-Δ3. The strength of the interaction was classified by the time taken for colonies to visibly turn blue in a β-galactosidase filter assay: +++, <25 min.; ++, 25-50 min; +50-100 min; -, no color change by 100 min.; and ND, not determined. *B.* The α-actinin-4 protein contains four major domains; N-terminal actin binding domain denoted as a gray diamond that interacts with F-actin, a central region containing spectrin-like repeats (denoted as striped ovals), C-terminal EF-hands (denoted by stippled ovals) are calcium binding domains that regulate α-actinin's actin binding capabilities. Finally, α-actinin-4 contains the C-terminal type I PDZ consensus sequence ESDL.

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Interaction of Rhophilin-2 with α-actinin-1 and α-actinin-4

In light of our yeast two-hybrid results and the fact that other α-actinin isoforms contain a consensus-binding motif for PDZ domains (ESDL) at their extreme C-termini, we examined and confirm whether full-length Rhophilin-2 could interact with either α-actinin-4 or α-actinin-1 (the two non-muscle α-actinins). To test these interactions we generated GST-fusion proteins using the last 120 amino acids of α-actinin-4 and α-actinin-1. This region of both molecules contains three of the four C-terminal EF-hand domains and the PDZ consensus sequence. Using these recombinant proteins left immobilized to glutathione beads we performed pull-down assays with lysates of Cos1 cells expressing FLAG-tagged Rhophilin-2. The interaction between Rhophilin-2 and α-actinin-4 was slightly stronger than the Rhophilin-2 interaction with α-actinin-1 (Fig. 5). In a similar assay, Rhophilin-1 was also able to interact specifically with GST-α-actinin-1 and GST-α-actinin-4 fusion proteins and not GST (Fig. 5). These results suggest that both Rhophilin-1 and -2 interact with the C-terminus of α-actinin-1 and -4.
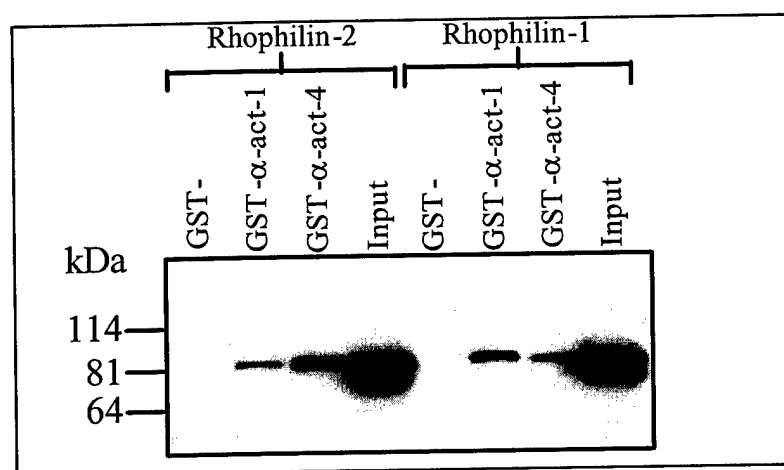
Fig. 5. Interaction of Rhophilin-1 and -2 with α-actinin-1 and α-actinin-4.
Cos1 cells were transfected with either Rhophilin-1 or Rhophilin-2 and then were incubated with GST, GST-α-actinin-1, and GST-α-actinin-4. Following Western blotting, bound FLAG-tagged Rhophilin-1 and -2 were detected with a mouse monoclonal antibody to the FLAG epitope, followed by goat anti-mouse horseradish peroxidase and detected by enhanced chemiluminescence.

Mapping of the region of Rhophilin-2 responsible for α-actinin-4 binding

In addition to testing full-length Rhophilin-1 and -2, we tested a variety of point and deletion mutants of Rhophilin-2 in order to map the interaction with α-actinin-4. One mutant, Rhophilin-2-Δ3, contains the same portion of Rhophilin-2 used in the yeast two-hybrid screen, was found to interact strongly with α-actinin-4 and less so with α-actinin-1. Another mutant tested, Rhophilin-2β □ contains an in-frame deletion of amino acids 399-587 within Rhophilin-2 and is missing the PDZ domain and approximately 100 amino before this region. Rhophilin-2β did not interact with either α-actinin isoform (data not shown). This result

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suggested the importance of the PDZ domain or the region preceding it in the Rhophilin-2-α-actinin interaction, which was not surprising as both α-actinin-1 and -4 contain a C-terminal type I PDZ domain recognition motif (ESDL). To confirm whether the PDZ domain was required for this interaction we tested Rhophilin-2-R518A,L526A,G527A, a triple point mutant in the PDZ domain previously shown to abolish the Rhophilin-2 induced disassembly phenotype (3). Much to our surprise this mutant not only retained binding, but seemed to interact better with both α-actinin-1 and -4 (data not shown). To further map the interaction, another mutant Rhophilin-2-Δ514 (amino acids 1-514) contains a C-terminal truncation and lacks the PDZ domain, was tested for binding. In this assay, Rhophilin-2-Δ514, lacking the PDZ domain, retained binding to α-actinin-4. An additional C-terminal truncation mutant, Rhophilin-2-Δ454 (amino acids 1-454), was also generated and tested in the *in vitro* binding assay. Once again, this mutant retained binding activity to α-actinin-4. Furthermore another C-terminal truncation mutant, Rhophilin-2-Δ420 (amino acids 1-420), was generated and tested in the *in vitro* binding assay. This construct however did not retain binding to α-actinin-4 (data not shown). These results suggest that the PDZ domain of Rhophilin-2 is not required for the Rhophilin-2-α-actinin interaction and that the interaction occurs in the region N-terminal to the PDZ domain, specifically somewhere between amino acids 420-454 of Rhophilin-2. Additional data base searches with this region do not show any obvious homology with other proteins besides Rhophilin-1.

Mapping the region of α-actinin-4 required for Rhophilin-2 binding.

In order to further explore the nature of the Rhophilin-2-α-actinin-4 interaction we wanted to map the region of α-actinin-4 required for binding. In addition to GST-α-actinin-4, two other mutants GST-α-actinin-4-C16 (amino acids 896-912), containing only the C-terminal 16 amino acids of α-actinin-4 (including the PDZ binding motif), and GST-α-actinin-4-ΔC (amino acids 1-908), missing the 4 C-terminal amino acids (the PDZ binding motif) of GST-α-actinin-4 where tested for binding with full-length Rhophilin-2 (data not shown). In this experiment Rhophilin-2 interacted with GST-α-actinin-4 and GST-α-actinin-4-ΔC, but not with GST or GST-α-actinin-4-C16. These results suggest that the interaction between Rhophilin-2 and α-actinin is not PDZ dependent because Rhophilin-2 did not interact with GST-α-actinin-4-C16, which retains the C-terminal PDZ binding motif. Additionally, Rhophilin-2 was still able to bind the GST-α-actinin-4-ΔC fragment that lacks the PDZ type I recognition motif leading us to believe that the Rhophilin-2-α-actinin-4 interaction may involve the EF-hands of α-actinin-4.

In light of the results demonstrating that the Rhophilin-2-α-actinin-4 interaction involved a region in α-actinin-4 containing EF-hand domains, we made additional mutations to the GST-α-actinin-4 protein to

further map the interaction. The GST-α-actinin-4 protein used in these experiments contains three of the four total EF-hand domains present in full-length α-actinin-4 (EF-hands 2,3, and 4). To further narrow the region responsible for the Rhophilin-2-α-actinin-4 interaction two additional mutants were tested, GST-α-actinin-4-EF2, containing only the second EF-hand of α-actinin, and GST-α-actinin-4-EF23, containing only the second and third EF-hands. When tested in the *in vitro* binding assay with Rhophilin-2 (data not shown), we found that the second EF-hand was sufficient for the interaction as Rhophilin-2. These results suggest that Rhophilin-2 can specifically interact with the second EF-hand domain of α-actinin and potentially EF-hand domains in other proteins.

Rhophilin-2 interacts with the EF-hand containing MRLC2.

While Rhophilin-2 was found to interact with a fragment of α-actinin-4 in yeast two-hybrid and *in vitro* with a fragment of α-actinin-1 and -4, immunoprecipitation experiments failed to show an interaction *in vivo* (data not shown). One likely reason is that in the native full-length molecule the EF-hand region is either masked or in an altered conformation that prevents Rhophilin-2 binding. Due to the requirement of the EF-hand domain in α-actinin-4 for the interaction with Rhophilin-2, we tested another actin regulatory protein that contains EF-hand domains, myosin regulatory light chain (MRLC2). Using the same affinity capture approach used with α-actinin-4, we found that Rhophilin-2 interacted with GST-MRLC2 (Fig. 6*A*). It should be noted that the GST-MRLC2 construct contains the full-length MRLC2 protein rather than a partial portion of the full-length protein as is the case with GST-α-actinin-4 (contains only the 120 C-terminal amino acids of full-length α-actinin-4).

Using immunoprecipitation experiments we looked for an association between full-length MRLC2 and Rhophilin-2 in intact cells. Cos1 cells were co-transfected with expression vectors encoding either Myc-tagged MRLC2 or Myc-tagged Snf7-1 and FLAG-tagged Rhophilin-2. Two days after transfection, lysates were prepared and Myc-tagged hSnf7-1 and MRLC2 were pulled down using anti-Myc antibody and Protein A/G-agarose beads. The bound proteins were then analyzed by Western blotting with anti-FLAG antibody to detect associated proteins (Fig. 6*B*). Rhophilin-2 bound to full-length MRLC2 but not to Snf7-1 (Fig. 6*B*). Since comparable amounts of FLAG-tagged Rhophilin-2 were present in the whole cell lysates (data not shown), these results indicate that the interaction of Rhophilin-2 is specific for MRLC2. These results also suggest that Rhophilin-2-MRLC2 interaction occurs *in vivo*.

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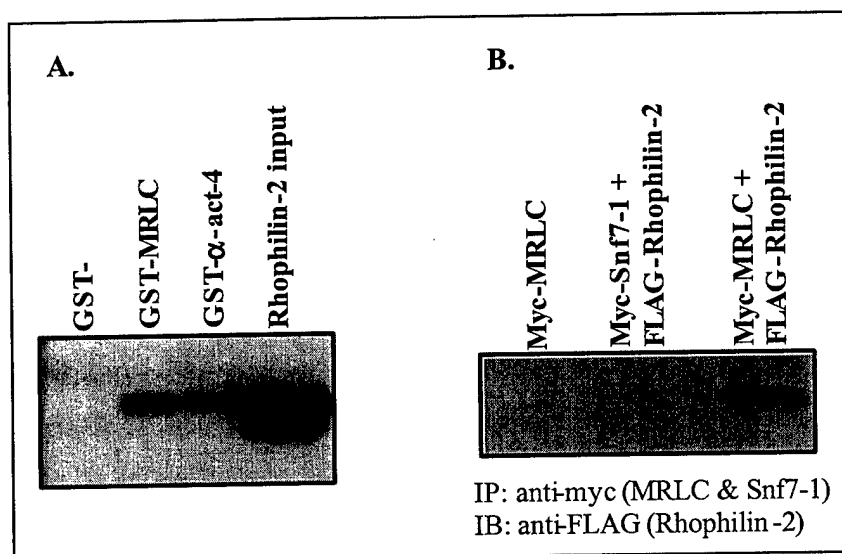
Fig. 6. Rhophilin-2 interacts with the EF-hand containing MRLC2

A. Cos1 cells transfected with FLAG-tagged Rhophilin-2 were incubated with GST, GST-MRLC2, and GST-α-actinin-4. Following Western blotting, bound FLAG-tagged Rhophilin-2 was detected with a mouse monoclonal antibody to the FLAG epitope, followed by goat anti-mouse horseradish peroxidase and detected by enhanced chemiluminescence. *B.* Myc-tagged MRLC2 and Snf7-1 were immunoprecipitated overnight with a mouse anti-Myc monoclonal antibody. Following immunoprecipitation samples were subject to Western blotting and probed with the M2 anti-FLAG™ monoclonal antibody. FLAG-tagged Rhophilin-2 specifically coimmunoprecipitates with Myc-MRLC2. Additionally, Rhophilin-2 does not interact with the Myc-Snf7-1 control.

Characterization of Endogenous Rhophilin-2 with a Polyclonal Antibody.

An extremely relevant reagent to understanding Rhophilin-2 function involves generating antibodies to Rhophilin-2. Using bacterially made recombinant protein for full-length Rhophilin-2, we have immunized rabbits for generating immune response to Rhophilin-2. Using the antisera, we have tested different cell lines for their Rhophilin-2 expression levels. As a positive control in Western blotting, we found that the antisera reacted with HeLa cells transfected with the Rhophilin-2 expression vector (69,000 Daltons). We have found that Rhophilin-2 is ubiquitous expressed in breast cancer cells such as MCF-7 cells and other normal tissues and cell types including muscle, Cos1 cells, and ovarian cancer cells (data not shown).

To gain further insight into its potential function, we have performed cell fractionation experiments and found that Rhophilin-2 exists as both a soluble and insoluble component (data not shown). Specifically, 1% Triton-X-100 did not completely solubilize Rhophilin-2, nor did rigorous extraction conditions with 6 M Urea or 0.1 N NaOH (data not shown). These results suggest that some of the Rhophilin-2 protein within cells is strongly associated with the cytoskeleton and another pool may be soluble.

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Studies with the Rho GTPase Effector Protein SPEC1.

I have further also characterized the role of other Rho GTPase effector proteins in cytoskeletal organization, shape changes and other signaling pathways. One protein that I studied was SPEC1, a small Cdc42-binding proteins of previously unknown normal function (4). Here, the regions of the SPEC1 molecule involved in Cdc42 binding, phosphoinositide binding and membrane targeting were mapped using deletion and point mutation analysis (data not shown). The Cdc42 binding activity of SPEC1 was altered by mutations within multiple regions of the molecule, including the N-terminus, the core CRIB sequence and a C-terminal alpha helical region. Phosphoinositide-binding region within SPEC1 was identified and mapped to a basic region preceding the CRIB sequence. These phospholipid-binding assays were used to determine that SPEC1, like N-WASP, binds to a variety of phosphoinositides. Overexpression studies of untagged SPEC1 protein in HeLa cells showed that SPEC1 was markedly enriched at the plasma membrane (Kisailus et al., manuscript in revision). Mutational analysis revealed that the plasma membrane association of SPEC1 required two cysteine residues (C10,C11) at the N-terminus. Overexpression of both wild type SPEC1 and Cdc42 induced membrane patches of colocalized SPEC1 and Cdc42 proteins, which was not observed with the SPEC1-C10A,C11A mutant. Collaborative studies have also shown that the related SPEC2 molecule is involved in T-cell function (Ching, Kisailus and Burbelo, submitted).

III. Key research accomplishments

*We have found that RL is a minor splice variant of Rhophilin-2.

*Rhophilin-2 overexpression disrupts adherens junctions of epithelial cells.

* GST-capture experiments show that Rhophilin-2 interacts with the EF-hand containing proteins including alpha-actinin and MRLC.

*Immunoprecipitition experiments show that MRLC2 interacts with Rhophilin-2 *in vivo*.

* Antibodies to Rhophilin-2 detect endogenous Rhophilin-2 numerous normal tissues and cell lines.

* Cell fractionation studies indicate that Rhophilin-2 exists as both a soluble and insoluble cellular component.

* Additional studies on the Rho GTPase effector protein SPEC1 show that it contains a phosphoinositide-binding region.

IV. REPORTABLE OUTCOMES

Degree: Received Ph.D. in Biochemistry and Molecular Biology (June, 2004). Presently I am Post-Doctoral

fellow doing cancer biology research in the Dr. Irwin Gelman, Roswell Park Cancer Institute, Buffalo, NY.

Publications:

Burbelo, P.D., **Kisailus, A.E.** and Peck, J.W. Detecting protein-protein interactions using *Renilla* luciferase fusion proteins. BioTechniques 33:1044-1050, 2002.

Kisailus, A.E., Ching, K., Pirone, D.M. and Burbelo, P.D. Cdc42 regulates diverse signaling pathways in mammalian cells. In Recent Advances in Cell Research, Research Signpost 1: 161-180, 2003.

Ching, K.H., **Kisailus, A.E.** and Burbelo, P.D., Recruitment of SPEC2 to the Immunological Synapse Journal of Immunology (in review, 2004).

Ching, K.H., Peck, J.W., **Kisailus, A.E.,** and Burbelo, P.D. Interaction of MRLC with Rhophilins (manuscript in preparation).

Kisailus, A.E., Ching, K.H., Foulkes, Pirone, D. M. and Burbelo, P.D. Structure and function of the SPEC1 protein (in revision, J. Biol. Chem., 2004).

Poster Presentations and Seminars:

Presented at poster session at an Era of Hope Meeting in Orlando Florida in September 2002

2002-2004 Seminars in the Department of Biochemistry and Molecular Biology, GUMC.

2002-2004 Poster presentation at the annual Georgetown Research fair, GUMC.

V. CONCLUSIONS
The data presented here provides insights into the normal function of the RhoA-binding protein Rhophilin-2. Much still needs to be learned about the role of Rhohphilin-2 as evident by a rescent publication showing that Rhophilin-2 interacts with a hCNK1, a regulator of Ras (5). Taken together these results suggest that Rhophilin-2 may act as a master integrator or scaffold connecting multiple signaling pathways and may normally function in a Rho pathway to limit stress fiber formation and/or increase the turnover of F-actin structures in the absence of high levels of RhoA activity.

From my Ph.D. studies funded by this DOD fellowship, I have learned a variety of molecular and cellular biology skills. I have worked in the area of Rho GTPases and this work has prepared me for my current position at the Roswell Park Cancer Institute in area of cancer cell biology. I have also worked on a second project more directly related to breast cancer cell motility and metastasis that I will get a first author research publication from this work.

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